Stability-Indicating HPLC–DAD Determination of Ribavirin in Capsules and Plasma

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A simple, selective and stability-indicating high-pressure liquid chromatographic method was developed for the analysis of ribavirin. Chromatographic separation was achieved by using a CPS Hypersil cyano column (4.6 × 250 mm, 5 μm particle size) with isocratic elution of the mobile phase, which was composed of 50 mM phosphate buffer, adjusted at pH 4 with phosphoric acid. The mobile phase was pumped at a flow rate of 0.8 mL/min. The detector was set at 240 nm and quantification of the analyte was based on peak area measurement. The method was validated with respect to linearity, range, precision, accuracy, selectivity, robustness, limit of detection and limit of quantitation. The calibration curve was linear in the range of 5–200 μg/mL with correlation coefficient > 0.999. Ribavirin was subjected to forced degradation studies under two conditions: mild and extensive stress testing. These studies included the effects of hydrolysis (neutral, acidic and alkaline) and oxidation, photolysis and dry heat. The proposed method proved to be stability-indicating by the resolution of the drug from its forced degradation products, making use of the diode array detector as a tool for confirmation of peak identity and purity. Moreover, the kinetics of alkaline degradation of ribavirin were investigated, an Arrhenius plot was constructed and the activation energy was calculated. The developed method was also extended to analyze ribavirin in capsules and in human plasma with good recovery values.

Introduction

Ribavirin (RBV), 1-[(2R, 3R, 4S, 5R)-3, 4-dihydroxy-5-((hydroxymethyl) oxolan-2-yl]-1H-1, 2, 4-triazole-3-carboxamide, is a synthetic nucleoside analogue structurally related to guanine. Its mode of action is still unclear; it may act at several sites, including cellular enzymes, to interfere with viral nucleic acid synthesis. It is used orally in the treatment of chronic hepatitis C, including HIV co-infection (1).

RBV is officially found in the United States Pharmacopeia (USP) 34 (2), which describes a high-pressure liquid chromatography (HPLC) method for its determination as bulk powder and as a solution for inhalation. It is also officially found in the British Pharmacopoeia (BP) 2010 (3), which describes a comparable HPLC method for its determination as bulk powder and nebulizer solution.

A search in the literature reveals that several spectrophotometric methods have been adopted for the determination of RBV in bulk and pharmaceutical preparations (4–7). Other reported methods for the assay of RBV in raw material or pharmaceuticals include: high-performance capillary electrophoresis (8), reversed-phase (RP)-HPLC with ultraviolet (UV) detection (9–12), polarimetry (13), infrared (IR) (14) and flow injection chemiluminescence (15), in addition to a stability-indicating thin-layer chromatographic (TLC) method, which was applied after subjecting the drug to different stress conditions (16).

Many publications concerning the determination of RBV in biological fluids can be found; the techniques adopted herein include: liquid chromatography–tandem mass spectrometry (LC–MS-MS) (17), solid-phase extraction (SPE) and HPLC (18), RP-HPLC (19–20) and radioimmunoassay (21). RBV has also been simultaneously determined with viramidine in human plasma by an LC–MS-MS method (22).

In the present work, RBV was subjected to forced degradation studies under two conditions: mild stress testing and extensive stress testing. These studies included the effects of hydrolysis (neutral, acidic and alkaline), oxidation, photolysis and heat (dry and wet).

The currently developed HPLC method effectively separated the drug from its degradation products under the two conditions. Moreover, the HPLC method proposed for RBV was utilized to investigate the kinetics of its alkaline degradation. An Arrhenius plot was constructed and the activation energy was calculated.

The developed stability-indicating HPLC method was used to analyze the drug in pharmaceutical preparations and in biological fluids such as plasma.

Although the literature contains some reports for the quantitation of RBV, only one method studied the degradation of the drug under stress conditions (16). Most mobile phases used in the available chromatographic procedures were composed of an aqueous phase and methanol as organic modifier (10, 11); few reports determined the drug in biological fluids (17–21). The proposed method demonstrates its novelty and merits by combining several advantages in one method: the limit of detection and quantitation were as low as 1.21 and 4.03 μg/mL, respectively; the method was able to determine the drug in both pharmaceutical preparations and plasma; the mobile phase consisted solely of phosphate buffer; also, stock, working and sample solutions were prepared in water. This suggests that the proposed method is cost-effective and environmentally friendly, which is especially important given that green chemistry has become a growing discipline.

Also, sample preparation involved only a simple procedure with no need for separation of the active pharmaceutical ingredient from the inactive additives. Another added advantage of the current method resides in its ability to study the kinetics of degradation and to separate RBV from different degradation products, which were obtained by subjecting it to stress conditions.
**Table I**

<table>
<thead>
<tr>
<th>Item</th>
<th>RBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept ( \beta )</td>
<td>5</td>
</tr>
<tr>
<td>Slope ( \alpha )</td>
<td>5</td>
</tr>
<tr>
<td>Regression equation:</td>
<td></td>
</tr>
<tr>
<td>( y = \beta + \alpha x )</td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient ( r )</td>
<td>0.999999</td>
</tr>
<tr>
<td>Variance ( (S_{y\cdot}) )</td>
<td>2.750.13</td>
</tr>
<tr>
<td>Accuracy (mean ( \pm SD ) (%)</td>
<td>98.73 ( \pm ) 0.52</td>
</tr>
<tr>
<td>Precision (RSD) (%)</td>
<td>0.27</td>
</tr>
<tr>
<td>LOD (( \mu g/mL ))</td>
<td>1.21</td>
</tr>
<tr>
<td>LOQ (( \mu g/mL ))</td>
<td>4.63</td>
</tr>
</tbody>
</table>

**Experimental**

**Apparatus**

The LC instrument was a Shimadzu model LC-20AD Prominence equipped with a SPD-M20A prominance photodiodearray (PDA) detector, connected to a computer loaded with Shimadzu software. A manual injector with a 20 \( \mu L \) loop was used. A Mettler Toledo MP 230 pH meter was utilized, in addition to a sonicator and a Sigma 2-16 centrifuge.

**Materials and reagents**

An authentic sample of RBV was provided by European Egyptian Pharmaceutical Industries and was used without any further purification. All chemicals and solvents were of HPLC grade. Phosphate buffer, pH 4, was prepared according to USP 34 (2) by using analytical grades of potassium dihydrogen phosphate, and pH was adjusted by using phosphoric acid. Plasma samples were donated from healthy volunteers.

**Stock solution**

A stock solution of RBV was prepared in purified water to obtain a final concentration of 5 mg/mL.

**General procedure**

**Development of the HPLC method and construction of the calibration curve**

The chromatographic separation was achieved by using a CPS Hypersil cyano column \((4.6 \times 250 \text{ mm} \times 5 \text{ m})\). The mobile phase was composed of 50 mM phosphate buffer of pH 4 (adjusted by using phosphoric acid). The mobile phase was filtered and degassed by passing through a 0.45 \( \mu m \) pore size membrane filter before use. The flow rate was 0.8 mL/min. The injection volume was 20 \( \mu L \) and the UV detector was set at 240 nm. All measurements were performed at ambient temperature.

The working solutions were prepared by dilution of the standard solution of the drug with the mobile phase to reach concentration ranges of 5–200 \( \mu g/mL \). (Table I). Triplicate injections were made for each concentration and chromatographed under the described HPLC conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration curve.

**Stability studies**

**Mild stress testing for RBV**

Aliquots of 5 mL from the RBV stock solution were transferred to 25 mL volumetric flasks, followed by 10 mL of purified water, and drops of each of the following solutions were added separately: 1 M HCl until pH 2 was reached, 1 M NaOH until pH 7, 1 M NaOH until pH 11 and 1 M NaOH until pH 7; this was followed by 0.5 mL of 30% \((v/v)\) hydrogen peroxide solution.

The volume was adjusted in each flask to 25 mL with purified water. Solutions 1–3 were kept at room temperature in the dark for 14 days, whereas Solution 4 was kept at room temperature in the dark for 24 h. After the specified time, Solutions 1 and 3 were neutralized to pH 7.0 and a 2 mL aliquot from each solution was diluted to 20 mL with the mobile phase and filtered before injection into the HPLC column. Chromatograms were recorded and compared to a standard solution of the same concentration without the previous treatment.

**Extensive stress testing for RBV**

**Oxidative degradation**

Oxidative degradation was performed by heating 5 mL from the RBV stock solution with 2 mL of 30% \((v/v)\) of hydrogen peroxide in a water bath at 80\(^\circ\)C for 30 min. After the specified time, the volume was adjusted to 25 mL by using purified water. A 2 mL aliquot from each solution was diluted to 20 mL with the mobile phase and filtered before injection into the HPLC column. Oxidative degradation was performed in the dark to exclude the possible degradative effect of light.

**Wet heat induced degradation**

An amount of the drug powder (2 g) was kept in an oven at 90\(^\circ\)C for 8 h. After the specified time, 100 mg of the powder were accurately weighed and dissolved in 20 mL of purified water. A 5 mL aliquot of this solution was diluted to 25 mL with purified water and a 2 mL aliquot was diluted to 20 mL with the mobile phase. The solution was filtered using a 0.45 \( \mu m \) filter before injection into the column.

**Dry heat induced degradation**

Heat degradation was performed by heating 5 mL from the RBV stock solution in a boiling water bath for 2 h in the dark. After the specified time, the volume was adjusted to 25 mL by
using purified water. A 2 mL aliquot from this solution was diluted to 20 mL with the mobile phase and filtered before injection into the column.

**Photolytic degradation**
The photodegradation study was performed by subjecting the stock solution of RBV to direct UV radiation (UV lamp set at 254 nm) at room temperature for 8 h. After the specified time, a 5 mL aliquot from the solution was diluted to 25 mL with purified water, and a 2 mL aliquot was diluted to 20 mL with the mobile phase and filtered before injection into the column.

**Kinetic investigation**

**Study of alkaline induced degradation of RBV at different temperatures**
From the stock solution of RBV, 5 mL aliquots were transferred to 25 mL volumetric flasks. Two and half milliliters of 0.5 M NaOH were added and the volumes were adjusted to 25 mL with purified water. The flasks were placed in a thermostated water bath, which was kept at different temperatures (55, 65, 75, 85 and 95 °C) for different time intervals (15–60 min).

At the specified time interval, 2 mL aliquots were neutralized by using 0.5 M HCl, diluted to 20 mL with the mobile phase and filtered before injection into the column.

The concentration of the remaining drug was calculated for each temperature and time interval. Data were further processed and degradation constants were calculated for each temperature to construct the Arrhenius plot.

**Application to pharmaceutical preparations**
The contents of 20 Ribavirin capsules (200 mg RBV/capsule) were mixed thoroughly. An amount of the powder equivalent to 400 mg of RBV was transferred to a 100 mL volumetric flask, extracted in 30 mL purified water and sonicated for 20 min, and the volume was adjusted with purified water. The solution was filtered. Aliquots of the filtrate were diluted with the mobile phase to suit the concentration range of the method, and filtered through a 0.45 mm membrane filter before injection.

The assay was also performed on expired Ribavirin capsules, which were obtained from a community pharmacy six months past their expiry date (Table II).

**Application to spiked human plasma**
The stock solution of RBV was diluted 100-fold with purified water. Different aliquots from the diluted stock solution (to suit the concentrations in Table III) were transferred into centrifugal tubes containing 4 mL of plasma. The tubes were shaken for 2 min by using a vortex tube shaker. Two milliliters of acetonitrile were added and the solutions were centrifuged for 30 min at 8,000 rpm. The centrifugates were transferred to clean centrifuge tubes and the residues were washed twice each with 1 mL of acetonitrile. The washing liquids were added to the centrifugates, which were diluted to 25 mL with the mobile phase, and the procedure was completed as described previously.

### Table II

<table>
<thead>
<tr>
<th>Item*</th>
<th>Proposed method</th>
<th>Comparative method (2)</th>
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</thead>
<tbody>
<tr>
<td>Valid Ribavirin capsules (200 mg RBV/capsule)</td>
<td>Recover (%)</td>
<td>101.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>104.41</td>
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<td></td>
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<td>103.88</td>
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<tr>
<td></td>
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<td>104.55</td>
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<tr>
<td></td>
<td>SD 1.18</td>
<td>0.66</td>
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<tr>
<td></td>
<td>t 0.405</td>
<td>1.37</td>
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<tr>
<td></td>
<td>F 3.17</td>
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<tr>
<td>Expired Ribavirin capsules (200 mg RBV/capsule)</td>
<td>Recover (%)</td>
<td>93.78</td>
</tr>
<tr>
<td></td>
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<td>93.18</td>
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<td></td>
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<td></td>
<td></td>
<td>95.30</td>
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<tr>
<td></td>
<td>SD 0.85</td>
<td>0.48</td>
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<tr>
<td></td>
<td>t 0.52</td>
<td></td>
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<tr>
<td></td>
<td>F 3.14</td>
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</table>

*Note: Theoretical values for t and F at p = 0.05 are 2.31 and 6.39, respectively.

### Table III

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Added (μg of RBV/mL of plasma)</th>
<th>Found* (μg of RBV/mL of plasma)</th>
<th>Recovery (%)</th>
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<tr>
<td>80</td>
<td>61.46</td>
<td>90.51</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>101.80</td>
<td>92.36</td>
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<tr>
<td>130</td>
<td>119.46</td>
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<tr>
<td>150</td>
<td>143.72</td>
<td>95.81</td>
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*Average of five determinations.

### Results

**Optimization of chromatographic conditions**

**Mobile phase composition and pH**
The best peak for RBV was obtained by using phosphate buffer–acetonitrile and a cyano column as a stationary phase. Concerning the ratio of the buffer to the organic solvent, the method was found to be robust for ratios of 100% buffer up to 60:40 (v/v). At a ratio of 50:50, a tailed peak was observed. Therefore, a mobile phase composed of 100% buffer was chosen for its potential in the stress testing to resolve the peak of the oxidation product from that of the parent drug.

Phosphate buffers of different pH values ranging from 2 to 8 in 0.5 unit increments were examined; no change in retention time or peak symmetry was observed for RBV throughout the studied range. Within the pH range of 3 to 5, the retention time remained constant and a sharp peak of sufficient symmetry was obtained; therefore, pH 4 was selected.

A buffer composed of 100 mg of metaphosphoric acid and 200 mg of potassium dihydrogen phosphate per liter was chosen for analysis. Changing the buffer concentration from half these values (50 or 100 mg) to 10-fold (1,000 or 2,000 mg) had no effect on retention times, peak shape or peak symmetry.

Figure 1 shows a typical chromatogram for RBV, which eluted at a retention time of 4.06 ± 0.071 min. The performance of the column (apparent efficiency) can be expressed by the number of theoretical plates (N), which equals approximately 1,677.
Stability-indicating aspects
Hydrolytic (using either HCl or NaOH solutions) and oxidative degradation (using H2O2) were conducted at both ambient temperature (mild stress testing) and by heating (extensive stress testing).

Effect of mild stress testing on ribavirin
RBV was stable under neutral conditions. However, under acidic, alkaline and oxidative conditions using mild conditions, reductions of approximately 7.5, 50 and 82%, respectively, were observed in the peak areas, with the appearance of additional small peaks attributed to degradation products that eluted at approximately 3.85 min for acidic degradation, 2.8 min for alkaline degradation and 3.15 min for oxidative degradation (Figure 2).

Effect of extensive stress testing on RBV
RBV was subjected to forced decomposition by acids, alkalis, heat, light and oxidation, as suggested in the International Conference on Harmonization (ICH) Q1A guideline (23). Alkaline and oxidative degradation resulted in 100% degradation, as evidenced by the disappearance of the parent drug peak and the appearance of degradation product peaks at 2.75 min for extensive alkaline degradation and 2.45 and 3.1 min for extensive oxidative degradation (Figure 3B and C). Acidic degradation resulted in 50% degradation of the parent drug and the appearance of a degradation product at 3.85 min (Figure 3A). No degradation was observed after exposure to dry heat, wet heat or UV light.

Peak purity tests obtained from PDA confirmed that RBV peaks were homogenous and pure in all samples subjected to stress conditions (Figure 4), thus confirming the stability-indicating power of the developed method.
Validation of the proposed method

Linearity and concentration range

The linearity of the proposed HPLC method was appraised by analyzing different concentrations for RBV. The linear regression equation was generated by least-squares treatment of the calibration data. Under the optimized conditions, the measured peak areas were found to be proportional to the concentrations of the drug. Table I shows the linear regression equation, concentration range, correlation coefficient, variance of the intercept ($S_a^2$), and variance around the slope ($S_b^2$). Satisfactory linearity is evident from the values of the correlation coefficient.

Accuracy

The accuracy of the proposed method was evaluated by calculating the recovery values of the drug added to regular excipients of tablets and capsules (lactose, microcrystalline cellulose, magnesium stearate, croscarmellose sodium and crospovidone), and good results were obtained (Table I). The accuracy was further confirmed by comparing the results of the assay of pharmaceutical preparations with the USP HPLC method (2) and by the Student’s $t$-test, which found no significant difference between the compared methods (Table II).

Precision

The repeatability of the proposed method was evaluated by calculating the relative standard deviation (RSD) of the assay results of three different concentrations, each in three replicates. The results are presented in Table I.

Selectivity

The selectivity of the proposed HPLC method was investigated by applying the chromatographic procedure to a solution of the co-formulated adjuvants for the dosage form (placebo). The chromatogram of this solution showed no peaks at the retention time of the analyte throughout the run time. The peak obtained for the capsule test solution was sharp and had a clear baseline separation (Figures 5 and 6). The chromatographic procedure was also applied to a solution containing a reference standard of RBV added to the solution of the co-formulated adjuvants (placebo). The chromatogram of this solution was exactly the same as that of the reference standard at the specified retention time. The obtained peak was sharp and had a clear baseline separation.

Further proof for the selectivity of the proposed HPLC method for the determination of RBV was conducted by testing the spectral purity of the eluted peak. The results indicated that the eluted peak was pure.

Limits of detection and quantitation

According to the pharmacopeial recommendations, the limit of detection (LOD) is defined as the concentration that has a
signal-to-noise ratio (S/N) of 3:1, whereas for the limit of quantitation (LOQ), the required S/N is 10:1.

The calculated LOD and LOQ values for the studied drug are shown in Table I.

Robustness
The robustness was evaluated by performing small variations in different conditions such as buffer pH (± 0.5 unit), working wavelengths (± 3 nm) and flow rates (± 0.1 mL/min). These variations had no effect on the measured responses.

Analysis of pharmaceutical preparations
The developed HPLC method was applied for the assay of RBV capsules. A representative chromatogram obtained from the dosage form solutions is shown in Figure 5. The diode array detector (DAD) allowed peak purity verification when no sign of co-elution from any of the inactive components was detected. Recovery values were calculated and the results revealed satisfactory accuracy and precision, as indicated from the recovery, standard deviation (SD) and RSD (Table I).

Expired RBV capsules were also analyzed and lower recovery values were obtained than for the valid capsules (Table II), which reinforced the need to conduct a stability study, especially when degradation products are unknown or not available (Figure 5B).

Determination of ribavirin in human plasma
The developed method was extended to the analysis of RBV in human plasma. Acceptable results were obtained, as evidenced by the recovery values, which ranged from 90.51 to 95.81% (Table III). The peak purity plot confirmed the absence of interference from endogenous components (Figures 7 and 8).

Alkaline-induced degradation kinetics for RBV
The degradation of RBV was conducted in 0.05 M sodium hydroxide at different temperatures (55, 65, 75, 85 and 95°C) to study the influence of temperature on the degradation process (Figure 9).

At the selected temperatures and sodium hydroxide concentrations, the degradation process followed pseudo-first order kinetics. From the slopes of the straight lines, it was possible to calculate the apparent degradation rate constant ($k_{obs}$), half-life ($t_{1/2}$) and shelf-life ($t_{90}$), as shown in Table IV. From the Arrhenius plot (Figure 10), the activation energy was calculated and found to be equal to 23.82 kcal/mol.

Discussion
The aim of this study during the method development was to achieve sufficient resolution between the intact drug peak and degradation product peaks with acceptable peak symmetry, within a reasonable analysis time. Several mobile phases were evaluated by using various proportions of different buffers and organic solvents.

Acetate buffer with either acetonitrile or methanol, and phthalate buffer with either acetonitrile or methanol, were tried on a C18 column, but the peak of the drug was found to co-elute...
with the solvent front. The best peak for RBV was obtained by using phosphate buffer of pH 4 and a cyano column as the stationary phase. Using these conditions, quantification was achieved by using DAD based on peak area measurement.

Forced degradation studies are described in various international guidelines. The ICH has published a set of guidelines that have been agreed upon and adopted by the American, European and Japanese regulatory authorities. The ICH guidelines that are applicable to forced degradation studies are: ICH Q1A, Stability Testing of New Drug Substances and Products; ICH Q1B, Photostability Testing of New Drug Substances and Products; ICH Q2B, Validation of Analytical Procedures: Methodology.

In ICH Q1A, there are recommended conditions for performing forced degradation studies on drug substances and products. The recommendations are to examine the effects of temperature (above that for accelerated testing, i.e., > 50°C), humidity (≥ 75% relative humidity), oxidation and photolysis. Testing in solution should also be performed across a wide pH range. These degradation studies are to be conducted to identify the likely degradation products of a drug substance, which in turn, can establish the degradation pathways and intrinsic stability of the molecules and validate the stability-indicating power of the proposed analytical procedure. In other words, according to ICH stability guideline Q1A (R2), stress testing is normally conducted under more severe conditions than those used for accelerated testing. However, such severe conditions that are typically applied may generate many irrelevant degradation products. Therefore, this study was also conducted under mild conditions, which would generate degradation products likely to be observed at the end of shelf-life or if the drug substance was not handled or stored properly; in other words, it was not considered desirable to generate samples with extensive degradation because of their limited relevance and because of the formation of secondary degradation products, which would lead to complicated degradation patterns (24).

The experimental investigation of the possible breakdown of drugs is usually studied to produce more stable preparations and to assist the pharmacists and physicians regarding the proper storage and handling of medicinal agents. Degradation reaction rates are affected by temperature, as suggested by the Arrhenius equation (25):

$$\log k = \log A - \frac{E_a}{2.303 RT}$$

In which $k$ is the specific reaction rate, $A$ is a constant known as the Arrhenius factor, $E_a$ is the energy of activation (the minimum kinetic energy a molecule must possess in order to undergo reaction), $R$ is the gas constant and $T$ is the absolute temperature.

In this study, the constants $A$ and $E_a$ were evaluated by determining $k$ for the alkaline degradation of ribavirin in 0.05 M NaOH at several temperatures and by plotting $1/\gamma$ against log $k$ (Figure 10). The resulting line was extrapolated at room temperature to obtain $k_{25°C}$, which is used as a measure of the stability of the drug under ordinary shelf conditions.

### Conclusion

In this study, a simple, selective and reliable HPLC–DAD procedure was developed for the assay of RBV in its pharmaceutical preparation and in human plasma.

The simplicity of the method was illustrated by the minimum requirements of chemicals and solvents, because the mobile phase consisted solely of phosphate buffer, and because stock, working and sample solutions were prepared in water. The selectivity was demonstrated by the ability of the method to separate the analyte from the forced degradation products obtained from both mild and extensive degradation conditions, and from formulation additives and plasma endogenous constituents. The reliability is guaranteed by the results of testing the various validation parameters of the method and the successful application to commercial dosage forms and plasma. The proposed method makes use of the DAD as a tool for peak identity and purity confirmation; however, it can be adapted to conventional HPLC with UV detection, which is available in quality control laboratories. Therefore, the proposed method can be recommended for routine analysis and for determining quality during stability studies of the drug.

### References


